

Sungthong, R. , van West, P., Heyman, F., Jensen, D. F. and Ortega-Calvo, J.-J. (2016) Mobilization of pollutant-degrading bacteria by Eukaryotic zoospores. *Environmental Science and Technology*, 50(14), pp. 7633-7640. (doi:[10.1021/acs.est.6b00994](https://doi.org/10.1021/acs.est.6b00994))

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Deposited on: 17 June 2016

1 **Mobilization of Pollutant-Degrading Bacteria by Eukaryotic**
2 **Zoospores**

3

4 **Rungroch Sungthong,^{†,||} Pieter van West,[‡] Fredrik Heyman,[§] Dan Funck**
5 **Jensen,[§] and Jose Julio Ortega-Calvo^{*,†}**

6 [†]Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC),
7 Apartado 1052, Seville 41080, Spain

8 [‡]Aberdeen Oomycete Laboratory, University of Aberdeen, Institute of Medical
9 Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

10 [§]Uppsala BioCenter, Department of Forest Mycology and Plant Pathology,
11 Swedish University of Agricultural Sciences, Uppsala 750 07, Sweden

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13 **Running title:** Enhancing Bacterial Mobilization by Zoospore Taxis

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15 **Word count:** 4876 words, 2 figures, 2 tables and 29 references cited.

16

17 **Keywords:** Eukaryotic zoospores; Pollutant-degrading bacteria; Mobilization;
18 Swimming behavior; Bioremediation

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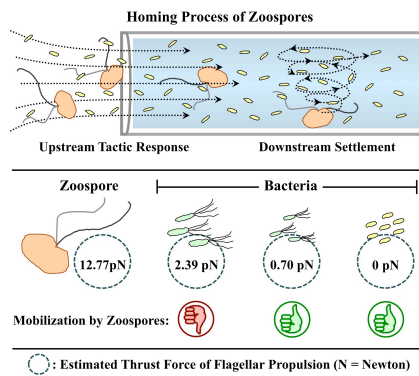
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26 **ABSTRACT:** The controlled mobilization of pollutant-degrading bacteria has
27 been identified as a promising strategy for improving bioremediation performance.
28 We tested the hypothesis whether the mobilization of bacterial degraders may be
29 achieved by the action of eukaryotic zoospores. We evaluated zoospores that are
30 produced by the soil oomycete *Pythium aphanidermatum* as a biological vector,
31 and, respectively, the polycyclic aromatic hydrocarbon (PAH)-degrading bacteria
32 *Mycobacterium gilvum* VM552 and *Pseudomonas putida* G7, acting as
33 representative non-flagellated and flagellated species. The mobilization assay was
34 performed with a chemical-in-capillary method, in which zoospores mobilized
35 bacterial cells only when they were exposed to a zoospore homing inducer (5%
36 (v/v) ethanol), which caused the tactic response and settlement of zoospores. The
37 mobilization was strongly linked to bacterial motility, because the non-flagellated
38 cells from strain *M. gilvum* VM552 and slightly motile, stationary-phase cells
39 from *P. putida* G7 were mobilized effectively, but the actively motile,
40 exponentially-grown cells of *P. putida* G7 were not mobilized. The
41 computer-assisted analysis of cell motility in mixed suspensions showed that the
42 swimming rate was enhanced by zoospores in stationary, but not in
43 exponentially-grown, cells of *P. putida* G7. It is hypothesized that the directional
44 swimming of zoospores caused bacterial mobilization through the thrust force of
45 their flagellar propulsion. Our results suggest that, by mobilizing
46 pollutant-degrading bacteria, zoospores can act as ecological amplifiers for fungal
47 and oomycete mycelial networks in soils, extending their potential in
48 bioremediation scenarios.

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72 INTRODUCTION

73 The microbial communities that are present at the solid-liquid interfaces of
74 polluted environments are often responsible for enhanced rates of pollutant
75 biodegradation, as compared with freely suspended communities.¹ Interface
76 communities usually start after mobilization or translocation of microbial cells to
77 the interface of the pollutant-containing matrices, which may be restricted by the
78 deposition and attachment of cells on adjacent surfaces along their travelling
79 distance.^{2,4} Self-propelled pollutant-degrading microbes (e.g., *Pseudomonas* spp.)
80 swim using their flagella, and they access pollutants through chemotaxis, which
81 has been recognized as a biological means for increasing pollutant bioavailability
82 and biodegradation.^{1,5} However, pollutant-degrading microbes that lack flagella
83 (e.g., *Mycobacterium* spp.) have other dispersal mechanisms, which may include
84 surface motility and/or gliding movements on moist surfaces.⁶ It is unclear how
85 these flagella-independent mechanisms contribute to overcome the restricted
86 bioavailability of pollutants, since these pollutant-degrading microbes have been
87 found to dominate specific microniches in polluted soils, such as those associated
88 to pollutant-enriched clay fractions.⁷ In any case, the directional mobilization of
89 pollutant-degrading microbes has been identified as a promising strategy for
90 improving bioremediation performance.¹

91 With an aim of improving microbial accessibility during bioremediation,
92 some chemical effectors have been found to modulate the motility behaviors of
93 self-propelled bacteria, leading to enhanced transport through porous media.^{3,4}
94 Nonetheless, little is known about the influence of biological effectors on bacterial
95 mobilization, e.g., other microbes that may co-exist with pollutant-degrading
96 bacteria. Some studies have reported mycelial networks of fungi and oomycetes

97 that have the capacity to provide water-saturated routes, facilitating the tactic
98 movement of flagellated polycyclic aromatic hydrocarbon (PAH)-degrading
99 bacteria towards PAHs.⁸⁻¹⁰ We recently showed that the zoospores that are
100 produced by the oomycete *Pythium aphanidermatum* can interact synergistically
101 with either flagellated or non-flagellated PAH-degrading bacteria in a set of
102 PAH-polluted microenvironments.¹¹ In that study, we determined that
103 PAH-degrading bacteria acted positively on zoospore development, for example,
104 by enhancing zoospore taxis to root exudates and diminishing the toxic influence
105 of PAHs on zoospore formation and taxis. Furthermore, the interactions between
106 zoospores and bacteria resulted in the initiation of complex biofilms at
107 pollutant-water interfaces. The enhancement of PAH bioavailability through
108 microbial colonization at pollutant-water interfaces by zoospore settlement,
109 germination and the formation of mycelial networks was therefore identified.
110 Despite these advancements, little is known about the mechanisms involved in the
111 dispersal of pollutant-degrading bacteria by eukaryotic zoospores.

112 For decades, scientists have been trying to understand the fluid mechanics
113 of microbial motion, in both quiescent and flowing regimes.¹²⁻¹⁵ The
114 physicochemical properties of fluids are used to interpret hydraulic activities, in
115 which the inertial-to-viscous forces ratio is one of the descriptive parameters in
116 fluid dynamics; this ratio is described by the Reynolds number (Re).¹²⁻¹⁵ The Re
117 value of a macroswimmer (for example a fish swimming in a river) is typically
118 much higher than 1, what correspond to the so-called “high- Re environments”.
119 The Re of the aqueous microenvironments surrounding microbial cells (an
120 example for a “low Re environment”)- is much lower than 1 for a fluid flow with
121 smooth and laminar motion at low velocities and small length scales.^{13,15} The

122 unique locomotion of self-propelled microbes within low-*Re* environments is
123 known to depend on their flagellar motors, which can cause dramatic changes in
124 flow. Some self-propelled microbes create thrust forces in front of their bodies
125 during swimming, and these microbes are known as “pullers” (e.g., biflagellate
126 algae). Microbes that create thrust forces behind their bodies are known as
127 “pushers” (e.g., bacteria).^{12,14} In bacteria, the thrust forces created by flagellar
128 propulsion ($f_{\text{propulsion}}$) are in the range of 0.1 – 1 pN (N = Newton).^{16,17} However,
129 the $f_{\text{propulsion}}$ values of eukaryotic zoospores and the impact of these forces on the
130 motion and mobilization of bacterial cells have yet to be known. The estimation of
131 these forces in microswimmers may offer an interpretation of the physical
132 interactions in connection with bacterial dispersal. Other mechanisms that may be
133 involved in biomobilization are changes in the fluid viscosity surrounding the
134 microenvironments of swimmers,^{18,19} or the direct association with the vector
135 organism.²⁰ However, swimming interactions in eukaryotic
136 microswimmer-bacteria mixtures within low-*Re* environments and their relevance
137 to the mobilization of bacterial cells have yet to be shown.

138 With the goal of ecological applications for innovative bioremediation
139 strategies, we have examined the possible role of *P. aphanidermatum* zoospores
140 as a biological vector for mobilizing two representative PAH-degrading bacteria
141 (*Pseudomonas putida* G7 and *Mycobacterium gilvum* VM552). Differences in the
142 motility of zoospores and PAH-degrading bacteria were computed and discussed
143 in relation to the mobilization of bacterial cells and the flow regime in the
144 mobilization assay. The effectiveness of the bacterial mobilization that was
145 caused by physical interactions with zoospore taxis was assessed by numerical
146 estimations using motility data.

147 MATERIALS AND METHODS

148 **Microbial Strains, Cultivation and Preparation.** The oomycete *P.*
149 *aphanidermatum* was used as a source of zoospores. The primary stock was
150 supplied by the Aberdeen Oomycete Laboratory at the University of Aberdeen,
151 UK. The oomycete was grown on diluted V8 (DV8) agar, and zoospore formation
152 was induced according to a protocol that is described elsewhere.¹¹ With this
153 protocol, $10^4 - 10^5$ zoospores mL⁻¹ in zoospore-forming solution (sterilized lake
154 water collected from Embalse Torre del Águila, Seville, Spain) were obtained.
155 The zoospore sizes were determined using a phase-contrast Axioskop 2 Carl Zeiss
156 microscope (Jena, Germany) with a 40×/NA0.65 A-plan objective (Carl Zeiss,
157 Germany) and connected to a Sony Exwave HAD color video camera (Sony,
158 Japan) and are given in Table 1.

159 The multiple PAH-degrader *M. gilvum* VM552 was supplied by D.
160 Springael (Catholic University of Leuven, Belgium), and the
161 naphthalene-degrader *P. putida* G7 was supplied by C.S. Harwood (University of
162 Washington, USA). Both bacterial strains were cultured in mineral salt media
163 supplemented with phenanthrene (Sigma-Aldrich, Germany) for *M. gilvum*
164 VM552²¹ or naphthalene (Sigma-Aldrich, Germany) for *P. putida* G7.^{3,4} These
165 bacterial cultures were then preserved in 20% (v/v) glycerol at -80°C and used as
166 a primary stock. For the mobilization assays, both bacterial strains were grown in
167 tryptic soy broth (Sigma-Aldrich, Germany) and incubated at 30°C with
168 reciprocal shaking at 150 rpm. *M. gilvum* VM552 cells were collected in the
169 exponential phase (~96 h of incubation). *P. putida* G7 cells were collected in the
170 exponential (~12 h of incubation) and stationary (~96 h of incubation) phases. The
171 initial densities of bacterial cells that were suspended in the sterilized lake water

172 were adjusted to an optical density ($OD_{600\text{ nm}}$) of 1.5. This OD corresponded to
173 10^{10} and 10^8 colony-forming units (CFU) mL^{-1} for *P. putida* G7 and *M. gilvum*
174 VM552, respectively.¹¹ The cell sizes of bacteria that are approximately 10 times
175 smaller than the zoospores, are shown in Table 1.

176 **Mobilization Assay.** A modified chemical-in-capillary method (Supporting
177 information, SI Figure S1A) was used to investigate the bacterial mobilization
178 caused by zoospores.¹¹ The chemical-in-capillary method is commonly used for
179 assaying the positive chemotaxis of self-propelled microbes. The level of
180 chemo-attraction performed by the microbes is determined by the difference in
181 viable counts detected in the capillary tubes that are filled with the test solution,
182 and subsequently connected to a chamber filled with the microbial suspension. In
183 this study, we adapted that method by using 5% (v/v) ethanol in the capillaries as
184 an inducer for zoospore homing, that is a set of sequential behaviors, comprised of
185 upstream swimming towards the inducer and downstream settlement (involving
186 the release of flagella and encystment).^{11, 22} Microbial suspensions were prepared
187 either with individual suspensions of bacterial cells or with pairwise mixtures of
188 both microbes. The bacterial cell suspensions were prepared by making a 10-fold
189 dilution relative to the initial density ($OD_{600\text{ nm}} = 1.5$) using sterilized lake water,
190 and the mixtures were prepared by diluting the zoospore suspensions. The final
191 density of zoospores in these experiments was $10^4 - 10^5$ zoospores mL^{-1} , and the
192 densities of the bacterial cells were 10^9 and 10^7 CFU mL^{-1} for *P. putida* G7 and *M.*
193 *gilvum* VM552, respectively. We estimated from the literature and own
194 experiments¹¹ that these cell densities would be realistic to use for simulating a
195 natural situation. There was observed no antagonism between the zoospores and
196 bacteria at these cell densities.¹¹ The formation of oxygen gradients would have

197 interfered, as a consequence of aerotaxis, with the measurements. However, this
198 can be excluded because the low concentration of dissolved organic carbon in the
199 solutions (9 mg L^{-1})¹¹ minimized the consumption of oxygen in the chamber.
200 Furthermore, we did not observe any microbial accumulation at the air-liquid
201 interfaces along the edge of the chamber, what would have unequivocally
202 indicated an aerotaxis reaction. The prepared microbial suspension ($\sim 500 \text{ }\mu\text{L}$) was
203 introduced to a chamber (depth = 1.09 mm, Figure S1A), where the open-ended
204 1- μL capillary tubes (inner diameter = 0.20 mm, Microcaps, Drummond,
205 Broomall, PA, USA) filled with the zoospore homing inducer^{11, 22} or sterilized
206 lake water (as a control) were inserted. Steady flow through the capillaries
207 allowed zoospore motility and settlement inside the capillaries. The inducer was
208 prepared by diluting absolute ethanol (Panreac, Barcelona, Spain) with sterilized
209 lake water, and the concentration (5% v/v) was chosen on the basis of the distance
210 of zoospore travel into the capillary tubes (SI Figure S1B) and the lack of
211 influence of this concentration on the bacterial cell viability (SI Figure S2A). The
212 chambers were incubated at 25°C for ~ 1 h. The homing process of the zoospores
213 was determined by recording the numbers of zoospore cysts inside the capillary
214 tubes. The capillary tubes were then taken out of the chamber, and their outer
215 walls were cleaned three times with sterilized distilled water. The whole liquid
216 volume (1 μL) inside each capillary tube was immediately transferred with a bulb
217 dispenser into a known volume of sterilized lake water for a serial dilution. The
218 capillary tube-connected dispenser was washed with the dilution solution for at
219 least 3 times to ensure the complete transfer of microbial cells. The number of
220 bacterial cells that entered the capillary tube ($\text{CFU } \mu\text{L}^{-1}$) was quantified after the
221 dilutions were developed on tryptic soy agar (Sigma-Aldrich, Germany)

222 supplemented with 0.3 g L⁻¹ cycloheximide, which prevented oomycete growth.
223 There was no influence on the viability of both bacterial strains from this
224 cycloheximide dose (SI Figure S2B).

225 **Physicochemical Properties and Hydraulic Activities of Fluids in the**
226 **Mobilization Assay.** There were two zones in the mobilization assay, including
227 1) the chambers that contained microbial cells suspended in sterilized lake water
228 and 2) the connected open-end capillary tubes that contained sterilized lake water
229 or 5% (v/v) ethanol (SI Figure S1A). A steady flow through the tubes occurred as
230 a result of evaporation and capillary forces. In addition, a low concentration of
231 ethanol might have caused changes in the hydrodynamic properties (e.g., fluid
232 density, dynamic viscosity, fluid flow velocity, *Re* and friction force) of the fluid
233 bodies between the two zones. To exclude the possibility that the changes caused
234 by ethanol interfered with the mobilization assay, we estimated the fluid density
235 and dynamic viscosity at the two zones using the Jouyban-Acree model (SI
236 Method S1).²³

237 We also measured the hydraulic flow rate (u_0) through the capillary tubes.
238 This value was calculated by determining the linear speed of spontaneously
239 flowing *M. gilvum* VM552 cells, which were used as a microbial tracer, at the
240 mid-depth of capillary tubes filled with 5% (v/v) ethanol. This measurement was
241 performed using the same microscope settings as described above. The focal plane
242 was set to 100 μ m below the inner wall of the capillary tube, as the mid-depth of
243 the capillary channel, to minimize the interaction of bacteria with surfaces.
244 Multiple motion records derived from the mobilization experiments were
245 processed with Windows Movie Maker, Microsoft Windows XP. Individual paths
246 were then selected randomly from the motion records and used for motion

analysis with the CellTrak program (version 1.5, Motion Analysis Corporation, CA, USA). Ten paths were used for calculations to plot the linear speeds as a function of the recording time. The u_0 value was calculated by linear regression. We assumed that the u_0 values detected at the mid-depth of the capillary tube corresponded to the maximum velocity of the fluid flow (u_{\max}) along the capillary channel in accordance to the parabolic velocity profile of the Poiseuille's law.

The Reynolds number (Re) was calculated using the equation

$$Re = \frac{\rho \cdot u_0 \cdot D_H}{\eta} \quad (1)$$

where u_0 is the hydraulic flow rate (in m s^{-1}), ρ is the fluid density in kg m^{-3} , η is the dynamic viscosity of the fluid in Pa s , and D_H is the inner diameter of the capillary tube in m ($0.20 \times 10^{-3} \text{ m}$).

Two friction forces, including the drag force of fluid motion (F_{drag}) and the thrust force of flagellar propulsion ($f_{\text{propulsion}}$) performed by each self-propelled microbe, were estimated in this study. Stokes' law was employed to estimate the value of F_{drag} that acted at the interface between a small spherical particle and a fluid. We assumed here that all microbial cells were nearly spherical particles. Hence, the F_{drag} in Newton (N) of the aqueous microenvironments that affected a single *M. gilvum* VM552 cell was estimated using the following equation:

$$F_{\text{drag}} = 6 \cdot \pi \cdot \eta \cdot R \cdot u_0 \quad (2)$$

where R is the radius of the spherical particles in m (assumed here to be half of the L/B ratio of *M. gilvum* VM552 in Table 1), and the other variables are described above. In low Re -environments, $f_{\text{propulsion}}$ can be described in the same way of F_{drag} .¹⁶ Therefore, $f_{\text{propulsion}}$ of each self-propelled microbe was estimated with Equation (2), where R was set as the half value of the cell length (Table 1),

271 and u_0 was the swimming speed of the microbe.

272 **Biomobilization Efficiency.** The mobilization efficiency of bacterial cells
273 by zoospores was estimated using the mobilization rate (M_{rate}) and the apparent
274 flow rate (u_Z). The M_{rate} value (in cells $\mu\text{L}^{-1} \text{s}^{-1}$ per zoospore) was calculated as

$$275 \quad M_{rate} = \frac{CFU_Z - CFU_0}{(N_Z - N_0) \cdot t} \quad (3)$$

276 where CFU_Z is the bacterial biomass (CFU μL^{-1}) that was mobilized in the
277 presence of zoospores and their homing inducer, CFU_0 is the bacterial biomass
278 (CFU μL^{-1}) mobilized at u_0 , N_Z and N_0 are the numbers of zoospore cysts formed
279 in the capillary tubes that contained the inducer and the sterilized water,
280 respectively, and t is the incubation time in s ($\sim 3,600$ s). Assuming that the
281 increased bacterial cell concentration in the capillary from the mobilization caused
282 by zoospores was accompanied by enhanced flow, the value of u_Z (in $\mu\text{m s}^{-1}$) was
283 calculated from the relative fraction of mobilized bacterial cells and the hydraulic
284 flow rate as follows:

$$285 \quad u_Z = u_0 \cdot \left[\frac{CFU_Z}{CFU_0} \right] \quad (4)$$

286 **Motion Analysis.** The same microbial suspensions that were used for the
287 mobilization assay were used for motion analysis. These determinations included
288 the swimming trajectory, speed, and rate of change of direction (RCDI). Only
289 flagellated microbes were included in the motion analysis. We first observed and
290 recorded the swimming behaviors of flagellated microbes using a phase-contrast
291 microscope connected to a video camera, described above. The focal plane was
292 also set to 100 μm below the inner wall of the capillary tube. Second, multiple
293 motion records derived from either the individual suspensions or the mixtures
294 were processed by cutting the records into 6 s-long segments. The longest

swimming paths were then selected randomly from the motion records and used for motion analysis with the CellTrak program. Four swimming patterns were assigned in this study: linear, circular, sine wave and tortuous. Example for these patterns are shown in SI Figure S3. The swimming speed ($\mu\text{m s}^{-1}$) and RCDI (deg s^{-1}) were computed under two-dimensional analyses, although upwards swimming action of zoospores was often observed at a rate $<1 \text{ s}^{-1}$ (data not shown). Both the speed and RCDI were normalized using the average values that were derived from the individual swimming paths and reported as the global speed and global RCDI, respectively.

Statistical Analysis. The mean value \pm standard deviation (SD) or standard error (SE) derived from any measurements were reported with the corresponding observation number. A comparison of multiple means was performed by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test in SPSS 16.0 (SPSS, Chicago IL, USA). The statistical results were described and reported with *F*-distributions, degrees of freedom and significant (*P*) values.

RESULTS

Bacterial Mobilization by Eukaryotic Zoospores. Zoospores mobilized *M. gilvum* VM552 cells only in the presence of the zoospore homing inducer (Figure 1A), as indicated by the significant difference in the number of bacterial cells entering the capillary tubes ($F_{(3, 10)} = 37.492$, $P < 0.0005$). A similar result was observed in stationary-phase *P. putida* G7 cells ($F_{(3, 10)} = 139.456$, $P < 0.0005$) (Figure 1E) but not with exponential-phase cells ($F_{(3, 16)} = 2.210$, $P = 0.127$) (Figure 1C). Along with these observations, the homing responses of zoospores to their inducer were confirmed by the significantly higher number of zoospore cysts

320 that were formed in the capillary tubes ($F_{(5, 17)} = 34.861$, $P < 0.0005$) (Figure 1B,
321 D and F). A set of control experiments showed no evidence for a tactic response
322 (both positive or negative) by *P. putida* G7 cells to the zoospore homing inducer
323 and zoospore cysts (SI Figure S5).

324 With the aim of discriminating the physicochemical and hydraulic
325 influences of the fluid bodies from the bacterial mobilization caused by zoospores,
326 we calculated the experimental hydraulic flow rate, using the non-flagellated *M.*
327 *gilvum* VM552 cells as microbial tracers (Figure 2 and SI Video S1;
328 The SI video files can be downloaded from the following link:
329 <http://digital.csic.es/handle/10261/96015>). The individual motion speeds were
330 plotted against the measurement times, and the resulting regression equation had a
331 slope close to 0, thus indicating a steady flow ($\partial u_0 / \partial t \approx 0$) in addition to a flow
332 rate of $19.51 \mu\text{m s}^{-1}$. Although these estimations were performed with
333 ethanol-containing capillaries, the flow rate was assumed to be the same in the
334 ethanol-free controls. This assumption was supported by the absence of
335 significant differences in the number of *M. gilvum* VM552 cells that were
336 mobilized in the absence of zoospores (Figure 1A, Control). The negligible
337 influence of ethanol on the hydrodynamic properties of the solutions that were
338 introduced into the capillaries was also confirmed by calculating their fluid
339 density, dynamic viscosity, Re , F_{drag} and $f_{propulsion}$ (SI Table S1). For example, Re
340 remained at very similar values with and without ethanol, i.e., 4.4×10^{-3} and $3.7 \times$
341 10^{-3} , respectively.

342 Using the numerical data derived from the mobilization assay (Figure 1), we
343 estimated the efficiency of bacterial mobilization caused by zoospore taxis by
344 determining the mobilization rate (M_{rate}) and the apparent flow rate (u_z) in the

345 experiments with *M. gilvum* VM552 and stationary-phase *P. putida* G7 cells. The
 346 results for the *P. putida* G7 cells in exponential phase were not included in these
 347 calculations because no significant differences were found between their CFU_Z
 348 and CFU₀ values. The M_{rate} values were very similar for the two bacterial species
 349 (24 cells $\mu\text{L}^{-1} \text{s}^{-1}$ per zoospore for *P. putida* G7 and 22 cells $\mu\text{L}^{-1} \text{s}^{-1}$ per zoospore
 350 for *M. gilvum* VM552). These mobilization activities led to u_Z values of 45.36 μm
 351 s^{-1} for *M. gilvum* VM552 and 87.88 $\mu\text{m} \text{s}^{-1}$ for stationary-phase *P. putida* G7 cells,
 352 which were two- and four-fold higher, respectively, than the hydraulic flow rate
 353 (19.51 $\mu\text{m} \text{s}^{-1}$). The differences in u_Z values between the two species, having a
 354 similar cell size (Table 1), suggest different hydrodynamic properties of
 355 stationary-phase *P. putida* G7 cells possessing immotile or slightly active flagella.

356 **Swimming Behaviors and Physical Interactions in Microbial**
 357 **Suspensions.** Given the significant differences in size and motility between
 358 zoospores and bacterial cells (Table 1), the swimming capabilities and interactions
 359 between both microbes might explain the mobilization activities observed in this
 360 study. In fact, both the bacteria and zoospores exhibited significant changes in
 361 their swimming behaviors in the mixed suspensions (Table 2). Randomly selected
 362 swimming trajectories of *P. putida* G7 cells and zoospores are displayed in SI
 363 Figures S6 and S7, respectively. In the absence of zoospores, exponential- and
 364 stationary-phase *P. putida* G7 cells showed dissimilar swimming behaviors. The
 365 stationary-phase cells swam at significantly lower speeds and higher RCDI than
 366 did the exponential-phase cells. The tortuous movement of both bacterial cell
 367 types increased significantly in the presence of zoospores, but the global
 368 swimming speed increased significantly only for stationary-phase cells. As a
 369 result, the value of $f_{propulsion}$ also increased. The global speed of stationary-phase *P.*

370 *putida* G7 cells remained at a significantly lower value than that of the zoospores
371 (approximately $80 \mu\text{m s}^{-1}$). However, when scaled to body lengths (Table 1),
372 global speeds of bacteria were significantly higher. The exponential-phase *P.*
373 *putida* G7 cells impacted the swimming behaviors of zoospores to a greater extent
374 than did the other bacterial cells, as evidenced by the significant decreases in
375 global speed and RCDI. The relative differences between the $f_{\text{propulsion}}$ values for
376 zoospore and bacterial swimming were the highest with stationary-phase *P. putida*
377 G7 cells. No significant attachment of bacterial cells to zoospores was observed in
378 any mixed suspension.

379 These observations were related to the separated suspensions, which were
380 not exposed to any ethanol gradient. These findings would represent the motility
381 interactions that occurred in the primary chamber of the mobilization assay. A set
382 of motion records derived directly from the mobilization assay revealed a clear
383 pattern in the enhancement of bacterial mobilization by the homing responses of
384 the zoospores, which either swam inside the capillary tubes or engaged in
385 encystment, releasing their flagella (SI Video S2 and Figure S4). Circular
386 motion was a key swimming pattern that was often performed by zoospores prior
387 to their encystment (SI Video S3). In addition, swimming *P. putida* G7 cells were
388 found either inside or outside the capillary tubes (SI Video S4), showing the
389 negligible influence of the inducer on bacterial motility. This finding was in
390 accordance with the control experiments (Figure S5), with no effect on the CFU
391 counts in the capillaries.

392

393 **DISCUSSION**

394 We found that swimming zoospores caused the directional mobilization of

395 PAH-degrading bacteria. This only occurred in the presence of the zoospore
396 homing inducer. The response of zoospores to their inducer is initiated by
397 swimming towards the chemical gradient of the inducer, followed by settlement
398 on solid-liquid and air-liquid interfaces.^{11,22,24} In fact, the cumulative settlement
399 (so called auto-aggregation) of oomycete zoospores can occur through a
400 combination of chemotaxis and bioconvection mechanisms.²⁵ The bacterial
401 mobilization that was observed in our study resembles the mobilization or
402 transport of microscale loads that result from the tactic responses to light of *C.*
403 *reinhardtii*.²⁰ That study showed that *C. reinhardtii* cells swam by phototaxis at
404 speeds of $\sim 100 - 200 \mu\text{m s}^{-1}$ and transported attached microbeads for a maximum
405 distance of 20 cm. However, in our study we did not observe any significant
406 attachment of bacterial cells to zoospores, which indicates that the mobilization
407 occurred through a different mechanism.

408 Our results indicate that mobilization by zoospores was strongly linked to a
409 lack of bacterial motility. This finding suggests a mobilization mechanism related
410 to flow dynamics. Slightly motile and non-flagellated bacterial cells were
411 mobilized effectively by zoospores, but the actively motile cells were not
412 mobilized. In mono-specific suspensions and in zoospore and bacteria mixtures,
413 the observed bacterial swimming behaviors were consistent with the mobilization
414 assay results. The exponential-phase *P. putida* G7 cells swam actively, at a global
415 speed that was very similar to the swimming speed of zoospores (Table 2). This
416 high speed likely made the bacterial motion independent of the changes that were
417 caused in the fluid body by the swimming zoospores, because the global speeds of
418 the bacterial cells did not change in the mixed suspensions (Table 2). However,
419 the stationary-phase *P. putida* G7 cells swam at a slower speed, which increased

420 significantly in the presence of zoospores. This change may be related to the
421 mobilization observed in Figure 1, if we postulate that the slow bacterial motion
422 increased the susceptibility to zoospore mobilization. This finding would also
423 apply to the non-flagellated *M. gilvum* cells. An increased global speed would
424 facilitate dispersion, as observed previously with *P. putida* G7 cells that were
425 exposed to glucose.⁴ In that study, glucose consumption and overflow energy
426 dissipation resulted in hypermotility behavior and increased dispersion in
427 capillary assays, compared with those for the glucose-free controls.

428 The precise biophysical mechanism by which zoospores mobilized the
429 bacterial cells is unknown. However, the distribution and transport of bacterial
430 cells are often affected by the physicochemical properties and hydraulic activities
431 of the surrounding fluids,^{3,4,14} that may have changed as a result of zoospore
432 homing. The capillary force, liquid volatility and air pressures might reflect the
433 flow regime in our experimental system. On the basis of calculations with the
434 relevant physical parameters of the ethanol solutions and the results from the
435 mobilization experiments without zoospores, we excluded the possibility that the
436 presence of ethanol in the capillaries interfered physically in the mobilization
437 assays. Based on the estimated flow regime in the mobilization assay, the *Re*
438 values of the solutions present in the capillaries were much lower than 1, which is
439 characteristic of microswimmers.¹³ The values calculated here were of the same
440 order for single swimming cells, at 10^{-4} .¹⁵ Under these conditions, and considering
441 the relative differences in the cellular dimensions, swimming speeds and $f_{\text{propulsion}}$
442 of all microbes used (Table 2 and SI Table S1), the results can be explained by
443 postulating that the thrust force created during zoospore swimming mobilized the
444 bacterial cells. Indeed, swimming zoospores possessed the greatest $f_{\text{propulsion}}$, which

445 was, respectively, 50- and 20-fold higher than the inherent drag force of the
446 flowing fluids and $f_{\text{propulsion}}$ in stationary-phase *P. putida* G7 cells. However, some
447 self-propelled bacteria and algae can change the viscosity of their surrounding
448 liquids, and these changes are dependent on their cell density and swimming
449 mechanisms, occurring mainly at high cell densities (i.e., $> 10^{10}$ bacteria/mL).^{18,19}
450 Mobilization may have also been associated, in some extent, to viscosity changes
451 caused by the directional swimming of zoospores. It is also possible that the
452 unique swimming behaviors of zoospores that were observed here as a result of
453 their tactic responses and prior to their encystment and settlement could have
454 provided pathways for bacterial mobilization through jet-like fluid motion.¹⁵ For
455 example, the circular motion of zoospores could have acted as a microscale
456 vortexing mechanism. This phenomenon should be investigated further.

457 Our results show that zoospores can act as ecological amplifiers of fungal
458 and oomycete actions, and they can extend, in several aspects, the concept of
459 “mycelial pathways” for PAH-degrading bacteria.⁸⁻¹⁰ First, because those studies
460 were performed, possibly to highlight the role of mycelia in transport, with an
461 oomycete (*Pythium ultimum*), that is not normally producing zoospores. Second,
462 the mobilization observed may be of relevance for non-flagellated bacterial PAH
463 degraders, such as *Mycobacterium* species, which may constitute a significant
464 fraction of the functional microbiome in PAH-polluted environments.^{6,7} Although
465 they seem to be less well transported through mycelial pathways than
466 self-propelled bacteria,⁹ in our study the absence of motility was, in relative terms,
467 a positive factor for the biomobilization caused by zoospores. Finally, flagellated
468 (and therefore chemotactically active) bacterial groups, such as *Pseudomonas* and
469 *Achromobacter*, can be dispersed through their own chemotactic navigation along

470 mycelial pathways,⁸ but they could also be biomobilized by zoospores at the cell
471 growth phases when flagellar motility is limited or not existing.

472 It is at present unclear as to whether eukaryotic zoospores play a significant
473 role in biomobilization processes under natural conditions in polluted soils.
474 Oomycetes including species of *Pythium* and the closely related *Phytophthora* are
475 found in most soils and often in close association with organic material and plant
476 surfaces. Some are plant pathogenic, causing important plant diseases
477 (damping-off of seedlings, root rot etc.) or they can function in biocontrol
478 interactions. However, methods used in studying filamentous fungi from plant or
479 soil samples are normally not designed for detecting oomycetes – traditional
480 methods as dilution plating will mainly detect conidia-forming fungi and if special
481 selective media and procedures are not used will not reveal oomycetes. Next
482 generation sequencing methods are likewise biased, as they normally have been
483 focussing on the internal transcribed spacer region for determining fungal
484 community structures and the choice/design of primers is crucial for what
485 organisms will be revealed. Barcoding of *Pythium* species would require special
486 attention.²⁶ Both methods will normally reveal presence of organisms in terms of
487 species richness but not function and will not give information of the stage the
488 organism is present in (mycelium, conidia, resting structure, zoospores, etc.).
489 Possibly for these reasons, very little is known about what relative roles fungi and
490 oomycetes play in polluted areas. The closely related oomycete *Saprolegnia*
491 *delica* has, however, been repeatedly isolated from drainage water polluted with
492 heavy metals and it was shown to be involved in bioaccumulation of heavy
493 metals.²⁷ Other studies also report the presence of oomycetes in sites polluted by
494 heavy metals²⁸ and hydrocarbons.²⁹ Based on the knowledge from natural

495 ecosystems and managed soil systems, oomycetes are indeed having important
496 ecological functions and we believe this is the case also in biofilms in polluted
497 soils. Thus, we argue that the role of oomycetes may be overseen in studies of
498 eukaryotes in biofilm formation either due to methodological bias or because they
499 were not considered. The role of zoospores released from true fungi in
500 bioremediation might also be relevant to address in future research as they will
501 have different swimming behaviours, as compared to *Pythium* zoospores.

502 Our findings would suggest that the active production of motile propagules
503 from mycelial networks, with specific sensing mechanisms related to taxis and
504 settlement, should be considered when designing new inoculants composed of soil
505 fungi and oomycetes and pollutant-degrading bacteria, aimed at the improvement
506 of bacterial accessibility during bioremediation.

507

508 **ASSOCIATED CONTENT**

509 **Supporting Information**

510 (Method S1) Estimating fluid density and dynamic viscosity in the mobilization
511 assay; (Table S1) physicochemical properties and hydraulic activities of fluids in
512 the mobilization assay; (Figure S1) chemical-in-capillary method; (Figure S2)
513 bacterial growth in the presence of 5% (v/v) ethanol or cycloheximide; (Figure
514 S3) determination of swimming patterns in self-propelled microbes; (Figure S4)
515 effects of circular zoospore motion on bacterial mobilization; (Figure S5) control
516 experiment for tactic responses of *P. putida* G7 cells in exponential phase to
517 zoospore cysts and inducer; (Figure S6) swimming trajectories of zoospores;
518 (Figure S7) swimming trajectories of *P. putida* G7 cells; (Video S1) determination
519 of the flow velocity for a fluid body; (Video S2) mobilization of bacterial cells by

520 zoospore taxis; (Video S3) circular motion and settlement of zoospores; (Video
521 S4) freely swimming *P. putida* G7 cells during the mobilization assay.

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523 **AUTHOR INFORMATION**

524 **Corresponding Author**

525 *Phone: (+34) 954 624 711; Fax: (+34) 954 624 002;

526 E-mail: jjortega@irnase.csic.es

527 **Present Address**

528 ^{||}Infrastructure and Environmental Research Division, School of Engineering,
529 University of Glasgow, Glasgow G12 8LT, UK

530

531 **Notes**

532 The authors declare no competing financial interest.

533

534 **ACKNOWLEDGMENTS**

535 This study was supported by the Spanish Ministry of Science and Innovation
536 (CGL2010-22068-C02-01 and CGL2013-44554-R), the Andalusian Government
537 (RNM 2337), and the CSIC JAE Program (RS). PvW has funding support from
538 the BBSRC and NERC. Thanks are also given to Sara Hosseini of the Uppsala
539 BioCenter, SLU, Uppsala, Sweden for a useful discussion on oomycete zoospores.

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- 626

627 **Table 1.** Sizes of Microbial Cells Used in This Study^a

Microbe	Length (μm)	Breadth (μm)	L/B ratio
<i>P. aphanidermatum</i>			
Zoospores (193)	17.78 ± 2.92	12.58 ± 2.35	1.43 ± 0.18
<i>M. gilvum</i> VM552 (51)	1.52 ± 0.46b	1.03 ± 0.11a	1.48 ± 0.45b
<i>P. putida</i> G7			
Exponential growth phase (50)	3.36 ± 0.83a	1.09 ± 0.11a	3.12 ± 0.82a
Stationary growth phase (50)	1.73 ± 0.40b	1.02 ± 0.10a	1.70 ± 0.36b

628 ^aThe numbers in parentheses indicate the number of observations. The length (L),
629 breadth (B) and L/B ratio are shown as the means ± SD. Lower-case letters refer
630 to significant differences in the lengths ($F_{(2, 148)} = 144.130$, $P < 0.0005$), breadths
631 ($F_{(2, 148)} = 6.484$, $P = 0.002$) and L/B ratios ($F_{(2, 148)} = 119.221$, $P < 0.0005$) among
632 the bacteria.

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647 **Table 2** Swimming Behaviors and Physical Interactions in Microbial Suspensions

swimming characteristics	<i>P. putida</i> G7			
	exponential-phase cells		stationary-phase cells	
	control	+ zoospores	control	+ zoospores
dominant trajectory (%)	circular (40.60) tortuous (37.40)	tortuous (97.60)	linear (49.40) tortuous (35.30)	tortuous (85.30)
global speed ($\mu\text{m s}^{-1}$)	$82.81 \pm 2.80\text{c}$	$74.54 \pm 1.55\text{c}$	$40.82 \pm 2.42\text{a}$	$56.37 \pm 2.09\text{b}$
global RCDI (deg s^{-1})	$264.39 \pm 18.17\text{a}$	$586.41 \pm 19.84\text{b}$	$485.71 \pm 27.61\text{b}$	$551.49 \pm 23.50\text{b}$
$f_{\text{propulsion}}$ (pN)	2.33	2.10	0.59	0.82
no. of observation	91	83	85	75
swimming characteristics	zoospores			
	control	+ <i>M. gilvum</i> VM552	+ <i>P. putida</i> G7	
			exponential-phase cells	stationary-phase cells
dominant trajectory (%)	tortuous (67.80%)	tortuous (50.85%) circular (47.46%)	tortuous (60.76%)	tortuous (70.37%)
global speed ($\mu\text{m s}^{-1}$)	$82.59 \pm 2.46\text{b}$	$88.97 \pm 2.68\text{b}$	$74.06 \pm 2.08\text{a}$	$86.38 \pm 1.82\text{b}$
global RCDI (deg s^{-1})	$772.90 \pm 41.73\text{d}$	$464.62 \pm 34.21\text{b}$	$256.28 \pm 12.55\text{a}$	$634.06 \pm 33.18\text{c}$
$f_{\text{propulsion}}$ (pN)	12.30	13.25	11.03	12.86
no. of observations	59	59	79	54

648 The global speeds and global rate of change of directions (RCDIs) are reported as
649 averages: the means \pm SE. The propulsion forces ($f_{\text{propulsion}}$) were estimated with
650 Equation (2). Lower-case letters refer to the significant differences in global
651 speeds ($F_{(3, 330)} = 68.597$, $P < 0.0005$) or global RCDIs ($F_{(3, 330)} = 43.511$, $P <$
652 0.0005) of *P. putida* G7 cells and in the global speeds ($F_{(3, 249)} = 9.926$, $P <$
653 0.0005) or global RCDIs ($F_{(3, 249)} = 60.243$, $P < 0.0005$) of zoospores.

Figure legends

Figure 1. Mobilization of bacterial cells by eukaryotic zoospores. *Mycobacterium gilvum* VM552 cells (A and B) and exponential- (C and D) and stationary-phase cells (E and F) of *Pseudomonas putida* G7 were used. A mobilization assay was performed in either the absence (control) or presence (+zoospores) of swimming zoospores and either in the absence (white bars) or presence (grey bars) of the inducer. The results are the means of at least triplicate experiments, where the error bars represent the SDs. Asterisks refer to significant differences in the means of bacterial (graphs A, C, or E) and zoospore (graphs B, D and F) counts within each experiment.

Figure 2. Determination of the flow regime in the mobilization assay. *Mycobacterium gilvum* VM552 cells that were flowing through a capillary tube filled with the zoospore settlement inducer were used to detect the flow velocities (u) of the fluid body by the CellTrak program. A model shows the detected locations of the flowing cells at different time points inside the capillary tube (32 mm length) (A). The flow velocities of the bacterial cells at the mid-depth plane of the capillary channel (32 mm length) were detected at different time points (u_x , t_x , where x is the point of detection). These detected flow velocities corresponded to the maximum flow velocity (u_{\max}) in the parabolic velocity profile of the Poiseuille's law. The results were plotted using the averaged mean velocities derived from ten bacterial cells that were detected at the same time, the error bars represent SDs, and the trending line (dash line) refers to the linear regression equation (B).

Figure 1.

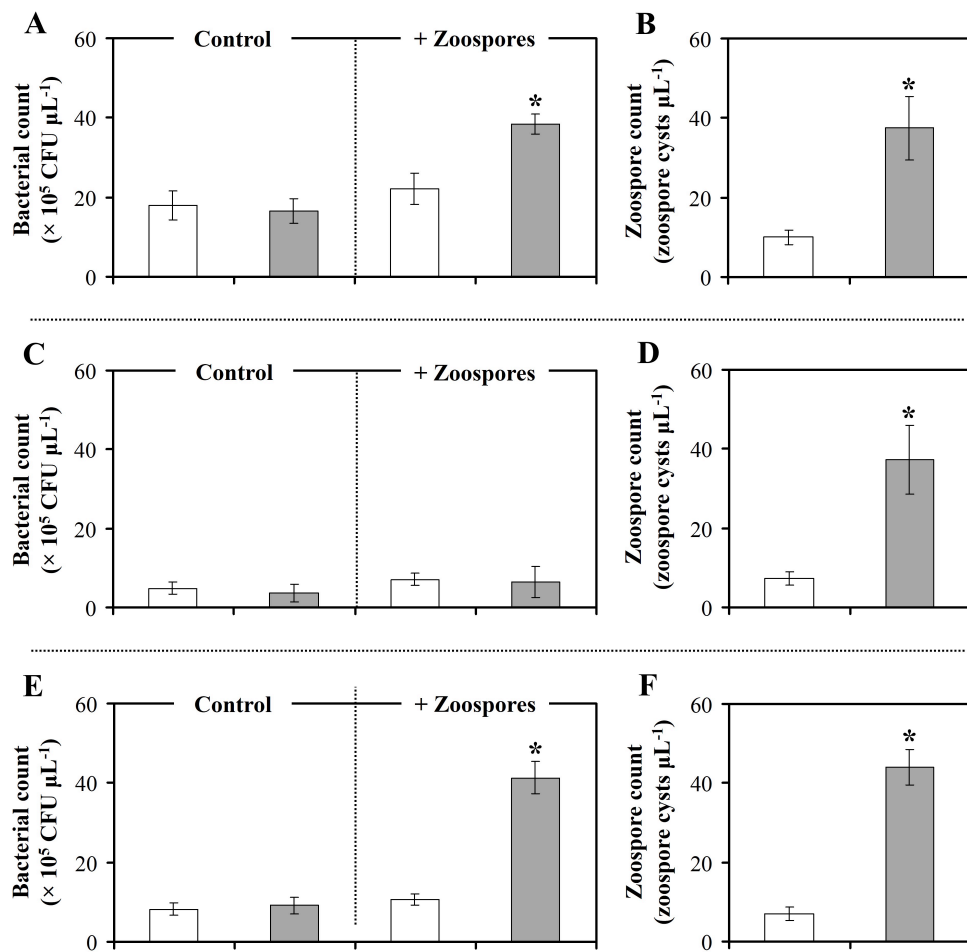


Figure 2.

